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Bisphosphonate esters interact with HMG-CoA reductase membrane domain to induce its degradation

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Abstract

HMG-CoA reductase (HMGCR) is a rate-limiting enzyme in the cholesterol biosynthetic pathway, and its catalytic domain is the well-known target of cholesterol-lowering drugs, statins. HMGCR is subject to layers of negative feedback loops; excess cholesterol inhibits transcription of the gene, and lanosterols and oxysterols accelerate degradation of HMGCR. A class of synthetic small molecules, bisphosphonate esters exemplified by SR12813, has been known to induce accelerated degradation of HMGCR and reduce the serum cholesterol level. Although genetic and biochemical studies revealed that the accelerated degradation requires the membrane domain of HMGCR and Insig, an oxysterol sensor on the endoplasmic reticulum membrane, the direct target of the bisphosphonate esters remains unclear. In this study, we developed a potent photoaffinity probe of the bisphosphonate esters through preliminary structure-activity relationship study and demonstrated binding of the bisphosphonate esters to the HMGCR membrane domain. These results provide an important clue to understand the elusive mechanism of the SR12813-mediated HMGCR degradation and serve as a basis to develop more potent HMGCR degraders that target the non-catalytic, membrane domain of the enzyme.

Keywords

HMG-CoA reductase; Degradation; SR12813; SR45023A (apomine); Bisphosphonate esters; Photoaffinity labeling

1. Introduction

HMG-CoA reductase (HMGCR) is a rate-limiting enzyme in the cholesterol biosynthetic pathway, and is responsible for the conversion of 3-hydroxymethyl-glutaryl CoA to mevalonate, which serves as an essential intermediate in synthesizing cholesterol. [1] Inhibitors of this

enzyme, a class of small molecules called statins, have been widely used to lower plasma cholesterol levels, which is a key risk factor for cardiovascular diseases. Statins mimic the partial structure of HMG-CoA to fit into the catalytic pocket of the HMGCR, and shut down the catalytic activity of the enzyme, leading to the uptake of plasma low-density lipoprotein, which contains a large amount of cholesterol and its esters, into the cells.

Cholesterol is an essential lipid for cells, and therefore, its abundance within cells is tightly regulated via transcriptional and post-translational mechanisms. With less cholesterol, transcription of relevant genes, including HMGCR and low-density lipoprotein receptor, is upregulated via activation of a master transcriptional regulator, sterol-regulatory element binding proteins (SREBPs). [2] In addition to such transcriptional feedback mechanisms, cells are equipped with another post-translational mechanism that controls the stability of HMGCR. [3] Late-stage intermediates in the cholesterol biosynthesis, lanosterols, or oxygenated sterols induce rapid degradation of HMGCR protein, [4,5] constituting another layer of feedback regulation, fine-tuning cholesterol homeostasis. [6,7] Aside from the sterols, geranylgeranyl pyrophosphate, a nonsterol metabolite downstream of mevalonate, is also involved in controlling the endoplasmic reticulum (ER)-associated degradation of polyubiquitinated HMGCR, adding another layer of complexity to the network of feedback mechanisms. [8,9]

Sterol-induced degradation of HMGCR requires only the transmembrane, non-catalytic domain of HMGCR, and an ER-resident oxysterol sensor Insig protein. [10-13] Oxysterol-binding to Insig protein induces interaction of HMGCR and Insig, and Insig brings E3 ubiquitin ligases into proximity of HMGCR, leading to accelerated ubiquitination and proteasomal degradation of HMGCR. [14-16] Binding of oxysterols to Insig also triggers the transcriptional negative feedback pathway, via formation of ternary complex of Insig, SREBP, and SREBP-cleavage activating protein, which prevents proteolytic activation of SREBP and eventually inhibits transcription of HMGCR and other SREBP target genes. In contrast to oxysterols, lanosterols only trigger HMGCR degradation, and are postulated to act through a similar but distinct mechanism from that of oxysterols. [5] Considering the presence of sterol-sensing domain within the HMGCR membrane domain, [16,17] which shares sequence similarity with the sterol-sensing domains of sterol transporters Niemann-Pick type C1 [18-22] and Ptch1, [23,24] a plausible hypothesis is that lanosterols directly bind to the membrane domain of HMGCR to induce the formation of HMGCR-Insig-E3 complex and subsequent degradation. [25,26]

Similarly to lanosterols, a class of compounds with bisphosphonate esters, including **SR12813** and **SR45023A** (also known as apomine) (Fig. 1), has been shown to trigger accelerated degradation of HMGCR without affecting SREBP pathway activity. [27] They were first identified by their cholesterol-lowering effect, [28-30] and subsequent analyses revealed that the bisphosphonates-induced degradation also requires the presence of the membrane domain of HMGCR and Insig, suggesting the same mechanism as that of lanosterols. [31-33] Although the lack of SREBP inhibitory activity points to the possibility of direct binding of the bisphosphonate esters to the membrane domain of HMGCR, the direct interaction between the bisphosphonate esters and HMGCR is yet to be proven. To evaluate their interaction, we aimed at developing a photoaffinity probe of the bisphosphonate ester. As structure-activity relationship (SAR) of

SR12813 derivatives on HMGCR degradation activity has not been reported, we first performed a preliminary SAR study using a luciferase-based HMGCR degradation assay. [34] Through the SAR, we identified a highly potent derivative, **SRP3042**, and successfully developed a potent photoaffinity probe, **srpDHY** (Fig. 1). Using these compounds and the probe, we provide the first experimental evidence supporting the direct interaction between the bisphosphonate ester and the membrane domain of HMGCR.

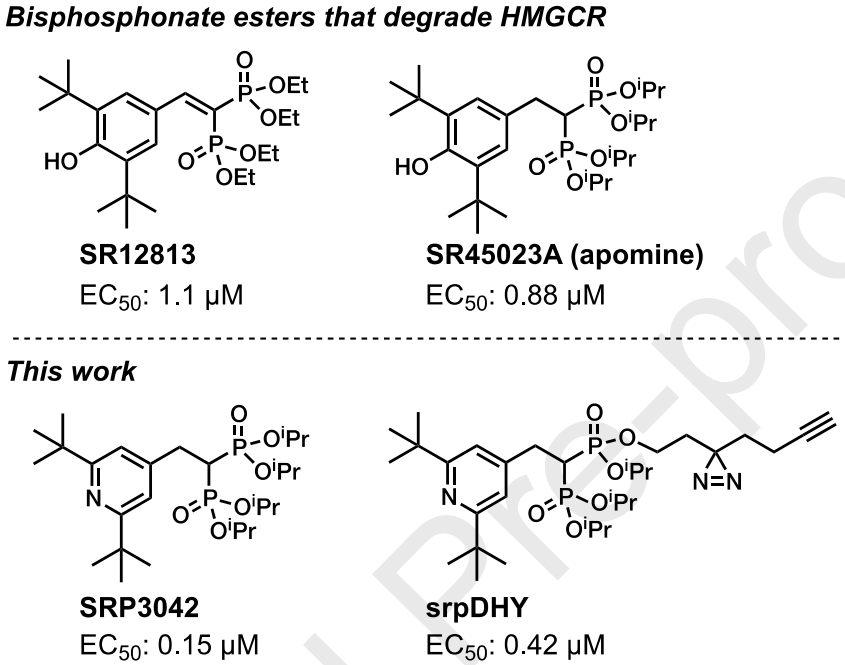


Figure 1. Bisphosphonate esters that induce degradation of HMG-CoA reductase (HMGCR). Here, we report **SRP3042** as the most potent HMGCR degrader known to date and the corresponding photoaffinity-labeling probe, **srpDHY**.

2. Materials and Methods

2.1 Materials

An expression vector (pCMV-Insig1-myc-DDK) that expresses human Insig1 tagged with myc and DDK (FLAG) under the control of the CMV promoter (Cat# RC200312, NCBI Reference Sequence NM_005542) was purchased from OriGene Technologies, Inc., Meryland, USA. Dulbecco's modified Eagle's medium (DMEM) was purchased from FUJIFILM Wako Pure Chemical Industries, Japan.

2.2 Cell lines and cell culture

HEK293 cells were obtained from American ATCC and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan). HEK293 cells stably expressing HMGCR-dCat-ELuc, the membrane domain of HMGCR

(residue number 1-499) fused with a FLAG tag and emerald luciferase, were maintained in the same medium in a humidified 5% CO_2 incubator as previously described. [34]

2.3 Synthesis of bisphosphonate esters

General methods. All chemical reagents and solvents were purchased from Sigma-Aldrich, Kanto Chemical Industry, and Wako Pure Chemical Industries, and used without further purification. Moisture-sensitive reactions were performed under an atmosphere of argon, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC; Merck silica gel 60 F254 plates). Bands were visualized using UV light, iodine vapors, acidic phosphomolybdic acid stain, and/or basic permanganate stain. Flash column chromatography was performed with silica gel (Silica gel 60 N, 40–50 μm particle size) purchased from Kanto Chemical. NMR spectra were recorded on a JEOL JNM-ECA500 spectrometer at 500 MHz for ^{1}H NMR, at 125 MHz for ^{13}C NMR, and at 202 MHz for ^{31}P NMR. Proton, carbon, and phosphorus chemical shifts are expressed in δ values (ppm) relative to internal tetramethylsilane (0.00 ppm) or residual $CHCl_3$ (7.26 ppm) for ^{1}H NMR, internal tetramethylsilane (0.00 ppm) or $CDCl_3$ (77.16 ppm) for ^{13}C NMR, and external triphenylphosphine (-5.65 ppm) for ^{31}P NMR. Data are reported as follows: chemical shift, multiplicity (s, singlet: d, doublet; t, triplet: q, quartet; m, multiplet: br, broad), coupling constants (Hz), and integration. High-resolution mass spectra were recorded using a Bruker micrOTOF II mass spectrometer. Room temperature or ambient temperature in the following synthetic procedures represents a range of temperature from 15 to 25 °C.

Tetraethyl 2-(4-hydroxyphenyl)ethenylidene-1,1-bisphosphonate (2). The Knoevenagel condensation of aldehydes and methylenediphosphonates was performed following the method reported by Lehnert et al. [35] To a solution of 4-hydroxybenzaldehyde **1** (248 mg, 2.03 mmol) in dry tetrahydrofuran (THF) (6 mL) was added $TiCl_4$ (0.69 mL, 6.1 mmol) slowly at 0 °C under Ar. Tetraethyl methylenediphosphonate (0.710 mL, 2.84 mmol) was added to the resulting mixture, followed by the dropwise addition of *N*-methylmorpholine (1.34 mL, 12.2 mmol) at 0 °C. The reaction was allowed to warm up to ambient temperature and stirred for 3 h. The reaction was cooled to 0 °C and quenched by adding ice tips, followed by saturated aqueous NH_4Cl solution. The mixture was extracted with EtOAc, and the organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/CH_2Cl_2/MeOH = 10:10:0 to 10:10:1) to afford the title compound (732 mg, 1.87 mmol, 92%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 9.64 (s, 1H), 8.24 (dd, *J* = 49.0, 29.5 Hz, 1H), 7.74 (d, *J* = 9.0 Hz, 2H), 6.90 (d, *J* = 9.0 Hz, 2H), 4.21–4.15 (m, 4H), 4.12–4.06 (m, 4H), 1.37 (t, *J* = 7.2 Hz, 6H), 1.24 (t, *J* = 7.2 Hz, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 162.80, 161.13, 133.83 (2C), 125.14 (dd, *J* = 21.6, 8.4 Hz), 115.64 (2C), 113.05 (dd, *J* = 173.9, 169.1 Hz), 62.73 (d, *J* = 6.0 Hz, 2C), 62.57 (d, *J* = 6.0 Hz, 2C), 16.26 (d, *J* = 6.0 Hz, 2C), 16.04 (d, *J* = 6.0 Hz, 2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 18.62 (d, *J* = 51.2 Hz, 1P), 13.82 (d, *J* = 51.2 Hz, 1P). HRMS–ESI (*m/z*): $[M - H]^-$ calcd for $C_{16}H_{26}O_7P_2$, 391.1081; found, 391.1093.

3-*tert*-Butyl-4-hydroxybenzaldehyde (4). To oxidize the methyl group on 2-*tert*-butyl-p-cresol, the DDQ/MeOH conditions were applied. [36] Note that the DDQ oxidation of this particular cresol has been reported to give poor yield of the aldehyde (< 5%), owing to oxidative coupling of the cresol. [37] A cobalt-catalyzed aerobic oxidation (1 mol% $Co(OAc)_2$, 1 atm O_2, 6 eq NaOH in ethylene glycol, 80 °C for 10 h) was also tested; however, the yield was quite low (4.2%).[38]

A solution of 2-*tert*-butyl-*p*-cresol **3** (386 mg, 2.35 mmol) in MeOH (3 mL) was treated with a solution of DDQ (1068 mg, 4.70 mmol) in MeOH (5 mL), and the resulting mixture was stirred at ambient temperature for 3 h. After evaporation of the solvent, the residue was suspended in $CHCl_3$/MeOH (10:1) and filtered through a silica gel pad to remove insoluble materials. Purification by flash column chromatography (hexane/EtOAc = 5:1 to 4:1) gave aldehyde **4** (76.4 mg, 0.429 mmol, 18%) as a pale brown oil. Rf = 0.25 (hexane/EtOAc = 2:1). ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 9.85 (s, 1H), 7.84 (d, *J* = 2.3 Hz, 1H), 7.64 (dd, *J* = 8.0, 2.3 Hz, 1H), 6.83 (d, *J* = 8.6 Hz, 1H), 6.28 (br s, 1H), 1.44 (s, 9H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 191.64, 160.41, 137.05, 130.09, 129.64, 129.61, 117.00, 34.70, 29.28 (3C).

Tetraethyl 2-(3-*tert*-butyl-4-hydroxyphenyl)ethenylidene-1,1-bisphosphonate (5). To a solution of **4** (85.2 mg, 0.478 mmol) in dry THF (4 mL) was added $TiCl_4$ (0.160 mL, 1.46 mmol) slowly at 0 °C under Ar. To the resulting mixture was added tetraethyl methylenebisphophonate (0.119 mL, 0.478 mmol), followed by dropwise treatment with *N*-methylmorpholine (0.315 mL, 2.87 mmol) at 0 °C. The reaction was allowed to warm up to ambient temperature and stirred for 18 h. The reaction was cooled to 0 °C, and quenched by adding ice tips followed by saturated aqueous NH_4Cl solution. The mixture was extracted with EtOAc, and the organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (EtOAc/CH_2Cl_2/MeOH = 100:100:2 to 100:100:5 to 100:100:10) to afford the title compound (46.8 mg, 0.104 mmol, 22%) as red oil. Rf = 0.25 (EtOAc/CH_2Cl_2/MeOH = 10:10:1). ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 9.46 (s, 1H), 8.25 (dd, *J* = 49.0, 29.5 Hz, 1H), 7.79 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.57 (d, *J* = 2.3 Hz, 1H), 6.89 (d, *J* = 8.6 Hz, 1H), 4.22–4.15 (m, 4H), 4.13–4.08 (m, 4H), 1.37 (t, *J* = 7.2 Hz, 6H), 1.37 (s, 9H), 1.24 (t, *J* = 6.9 Hz, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 163.79, 160.25, 135.80, 132.56, 130.91, 124.59 (dd, *J* = 21.6, 8.4 Hz), 116.84, 111.34 (dd, *J* = 174.5, 169.7 Hz), 62.63 (d, *J* = 6.0 Hz, 2C), 62.48 (d, *J* = 4.8 Hz, 2C), 34.67, 29.25 (3C), 16.29 (d, *J* = 7.2 Hz, 2C), 16.05 (d, *J* = 7.2 Hz, 2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 19.36 (d, *J* = 52.0 Hz, 1P), 14.34 (d, *J* = 52.0 Hz, 1P). HRMS–ESI (*m/z*): $[M - H]^-$ calcd for $C_{20}H_{34}O_7P_2$, 447.1707; found, 447.1722.

Tetraethyl 2-(3,5-di-*tert*-butylphenyl)ethenylidene-1,1-bisphosphonate (7). To a solution of 3,5-di-*tert*-butylbenzaldehyde **6** (218 mg, 1.00 mmol) in dry THF (2 mL) was added $TiCl_4$ (0.329 mL, 3.00 mmol), tetraethyl methylenediphosphonate (0.348 mL, 1.40 mmol), N-methylmorpholine (0.625 mL, 5.68 mmol) at 0°C under argon atmosphere. After stirring for 4 h, saturated aqueous NH_4Cl was added. THF was removed under reduced pressure. The residue was extracted with EtOAc and washed brine, combined organic layers were dried over Na_2SO_4,

and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel ($CHCl_3$/MeOH = 19:1) to afford the title compound **7** (142 mg, 0.291 mmol, 29%) as a colorless oil. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.33 (dd, *J* = 48.1, 29.2 Hz, 1H), 7.64 (d, *J* = 1.7 Hz, 2H), 7.47 (s, 1H), 4.27–4.15 (m, 4H), 3.95-4.08 (m, 4H), 1.38 (t, *J* = 7.2 Hz, 6H), 1.33 (s, 18H), 1.11 (t, *J* = 7.2 Hz, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 162.93, 150.64 (2C), 133.76 (dd, J = 22.2, 7.8 Hz), 125.31 (2C), 124.97, 119.21 (t, *J* = 168.5 Hz), 62.70 (d, *J* = 6.0 Hz, 2C), 62.30 (d, *J* = 7.2 Hz, 2C), 35.06 (2C), 31.45 (6C), 16.45 (d, *J* = 7.2 Hz, 2C), 16.04 (d, *J* = 7.2 Hz, 2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 18.71 (d, *J* = 54.5 Hz, 1P), 13.03 (d, *J* = 54.5 Hz, 1P).

Tetraethyl 2-(4-hydroxy-3,5-di-*tert*-butylphenyl)ethan-1,1-bisphosphonate (8). To a solution of **SR12813** (33.6 mg, 0.0666 mmol) in EtOH (1 mL) was added $LiBH_4$ (20.1 mg, 0.923 mmol) slowly at 0 °C, and the reaction mixture was stirred for 1 h at ambient temperature. Additional portion of $LiBH_4$ (24.2 mg, 1.11 mmol) was added at the temperature, and the bright yellow suspension was stirred for 16 h. The reaction was cooled to 0 °C, and quenched by adding saturated aqueous NH_4Cl solution. The colorless mixture was extracted with EtOAc, and the organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash silica gel column chromatography (EtOAc/CH_2Cl_2/MeOH = 50:50:1 to 10:10:1) to afford the title compound (21.0 mg, 0.0415 mmol, 62%) as colorless oil. Rf = 0.25 (EtOAc/CH_2Cl_2/MeOH = 10:10:1), slightly more polar than the starting material. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.07 (s, 2H), 5.08 (s, 1H), 4.14–4.04 (m, 8H), 3.17 (td, *J* = 16.6, 6.3 Hz, 2H), 2.64 (tt, *J* = 24.1, 6.3 Hz, 1H), 1.42 (s, 18H), 1.27 (t, *J* = 7.2 Hz, 6H), 1.25 (t, *J* = 7.2 Hz, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 152.29, 135.57 (2C), 130.08 (t, *J* = 7.2 Hz), 125.44 (2C), 62.46 (d, *J* = 6.0 Hz, 2C), 62.31 (d, *J* = 6.0 Hz, 2C), 39.47 (t, *J* = 131.4 Hz), 34.24 (2C), 31.05 (t, *J* = 4.8 Hz), 30.27 (6C), 16.29 (d, *J* = 7.1 Hz, 4C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 23.05. HRMS–ESI (*m/z*): $[M - H]^-$ calcd for $C_{24}H_{44}O_7P_2$, 505.2490; found, 505.2483.

Tetraisopropyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenylidene-1,1-bisphosphonate (10). To a solution of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde **9** (469 mg, 2.0 mmol) in dry THF (4 mL) was added $TiCl_4$ (0.658 mL, 6.0 mmol), tetraisopropyl methylenediphosphonate (0.910 mL, 2.8 mmol), and N-methylmorpholine (1.25 mL, 11.4 mmol) at 0°C under argon atmosphere. After stirring for 4 h at room temperature, saturated aqueous NH_4Cl was added, and THF was removed under reduced pressure. The residue was extracted with EtOAc, and the organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (EtOAc /Hexane = 4:1) to afford the title compound **10** (831.5 mg, 1.483 mmol, 74%) as a pale yellow solid. The spectra obtained were consistent with those in the previous report. [39] ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.20 (dd, *J* = 48.1, 30.4 Hz, 1H), 7.77 (s, 2H), 5.60 (s, 1H), 4.83–4.74 (m, 2H), 4.72–4.63 (m, 2H), 1.44 (s, 18H), 1.39 (d, *J* = 5.7 Hz, 6H), 1.35 (d, *J* = 6.3 Hz, 6H), 1.22 (d, *J* = 5.7 Hz, 6H), 1.16 (d, *J* = 5.7 Hz, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 161.29, 156.84, 135.58 (2C), 130.26 (2C),

125.92 (dd, *J* = 21.6, 8.4 Hz), 117.30 (t, *J* = 169.1 Hz), 71.25 (d, *J* = 6.0 Hz, 2C), 71.13 (d, *J* = 6.0 Hz, 2C), 34.69 (s, 2C), 30.43 (s, 6C), 24.17–24.27 (m, 6C), 23.76 (d, *J* = 6.0 Hz, 2C).

Tetraisopropyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethan-1,1-bisphosphonate (12). To a solution of tetraisopropyl methylenediphosphonate (347 mg, 1.01 mmol) in dry THF (5 mL) was added *n*BuLi (0.61 mL, 0.958 mmol) at -78 °C under argon atmosphere. After 10 min, to the solution was added dropwise a solution of 3, 5-di-*tert*-butylbenzyl bromide **11** (90.8 mg, 0.321 mmol) and tetrabutylammonium iodide (TBAI) (22.7 mg, 0.0615 mmol), and the reaction was stirred at room temperature for 16 h. The reaction mixture was diluted with water and extracted with EtOAc, and the organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The crude product was purified by flash column chromatography (EtOAc/CH_2Cl_2/MeOH = 100:100:0 to 100:100:1 to 100:100:5) to afford the title compound (126.5 mg, 0.231 mmol, 72%) as a colorless oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.25 (s, 1H), 7.10 (s, 2H), 4.79–4.70 (m, 4H), 3.21 (td, *J* = 16.5, 6.0 Hz, 2H), 2.59 (tt, *J* = 24.0, 6.0 Hz, 1H), 1.31–1.29 (m, 30H), 1.23 (d, *J* = 6.3, 6H), 1.20 (d, *J* = 6.3, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 150.36, 138.97 (t, *J* = 7.2 Hz, 2C), 123.17 (2C), 120.28, 71.11 (t, *J* = 3.6 Hz, 2C), 70.83 (t, *J* = 3.0 Hz, 2C), 40.89 (t, *J* = 133.8 Hz), 34.75 (2C), 32.00 (t, *J* = 4.8 Hz), 31.49 (6C), 24.17 (4C), 23.88 (m, 2C), 23.70 (m, 2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 21.05 (s, 2P).

Tetraisopropyl 2-(-2,6-di-*tert*-butylpyridine)ethan-1,1-bisphosphonate (SRP3042). Step 1. The intermediate **15** was prepared as reported previously, with slight modifications. [40] To a solution of 2,6-di-*tert*-buty-4-methylpyridine **13** (190 mg, 0.925 mmol) in dichloroethane (DCE) (3 mL) was added N-bromosuccinimide (NBS) (171 mg, 0.961 mmol, 1.04 eq) and 2,2'-azobis(isobutyronitrile) (AIBN) (30.3 mg, 0.187 mmol, 0.2 eq) at room temperature, and the reaction was heated to reflux for 1 h with a fluorescent light turned on. An additional portion of AIBN was added, and the reaction was further refluxed for 1h. Then the reaction mixture was cooled and diluted with hexane (9 mL). Precipitates was filtered off, and the filtrate was concentrated under reduced pressure to afford the crude 4-bromomethyl-2,6-di-*tert*-butylpyridine **14** (232 mg) as a colorless oil. This crude compound **14** was used in the next reaction without further purification. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.08 (s, 2H), 4.38 (s, 2H), 1.34 (s, 18H).

Step 2. To a solution of tetraisopropyl methylenediphosphonate (0.526 mL, 1.62 mmol) in dry THF (4 mL) was added *n*BuLi (1.55 M in hexane, 0.94 mL, 1.46 mmol) at -78 °C under argon atmosphere. After 10 min, a solution of the crude 4-bromomethyl-2,6-di-*tert*-butylpyridine **14** (230 mg) and TBAI (62.1 mg, 0.168 mmol) in dry THF (4 mL) was added dropwise. The reaction was stirred at room temperature for 16 h, and partitioned between water and EtOAc. The organic layer was separated, washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. Purification by flash column chromatography (EtOAc/CH_2Cl_2/MeOH = 100:100:1) gave the title compound (222 mg, 0.405 mmol, 44% over 2 steps) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 6.99 (s, 2H), 4.80–4.71 (m, 4H), 3.15 (td, *J* = 16.3, 6.3 Hz, 2H), 2.56 (tt, *J* = 24.1, 6.3 Hz, 1H), 1.33 (s, 18H), 1.31 (d, *J* = 6.3 Hz, 12H),

1.23 (d, *J* = 6.3 Hz, 6H), 1.22 (d, *J* = 6.3 Hz, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 167.30 (2C), 148.46 (t, *J* = 7.2 Hz), 116.01 (2C), 71.37 (t, *J* = 3.5 Hz, 2C), 71.06 (t, *J* = 3.5 Hz, 2C), 40.02 (t, *J* = 135.0 Hz), 37.50 (2C), 31.67 (t, *J* = 4.8 Hz), 30.17 (6C), 24.15 (4C), 23.92–23.86 (m, 2C), 23.76–23.71 (m, 2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 20.47 (s, 2P). Copies of the NMR spectra can be found in the Supplementary Materials.

3-Iodo-5-*tert*-butyltoluene (16). The title compound was prepared by following the reported procedure. [41] A 100 mL flask was charged with 3-iodotoluene **15** (10.87 g, 49.9 mmol) and 2-chloro-2-methylpropane (6.90 g, 74.5 mmol), and cooled to 0°C under argon atmosphere. To the cooled flask was added $AlCl_3$ (199 mg, 1.49 mmol), and the reaction was stirred at the temperature for 20 min. To the dark red solution was added ice tips, water, and saturated aqueous $NaHCO_3$, and the mixture was extracted with CH_2Cl_2. The dark purple organic layer was washed with saturated aqueous $Na_2S_2O_3$ and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The liquid was purified by vacuum distillation (7 mmHg, fractions collected between vapor temperature of 110-120°C) to afford the title compound (7.74 g, 28.2 mmol, 57%) as a faintly orange liquid which partially crystallizes upon cooling to 4 °C. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.50 (s, 1H), 7.36 (s, 1H), 7.13 (s, 1H), 2.29 (s, 3H), 1.28 (s, 9H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 153.42, 139.72, 135.15, 131.67, 125.64, 94.56, 34.60, 31.19, 21.20 (3C).

Tetraisopropyl 2-(3-iodo-5-*tert*-butyl-4-hydroxyphenyl)ethan-1,1-bisphosphonate (18). Step 1. To a solution of 3-Iodo-5-tertbutyl-methybenzene **16** (1.40 g, 5.10 mmol) in dichloroethane (10 mL) was added NBS (954 mg, 5.36 mmol, 1.05eq) and AIBN (167 mg, 1.02 mmol, 0.2eq) at room temperature, and the reaction was heated to refulx for 1 h with a fluorescent light turned on. An additional portion of AIBN was added, and the reaction was further refulxed for 1.5 h. Then the reaction mixture was cooled. Precipitates was filtered off, and the filtrate was concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (EtOAc/Hexane = 1:20) to afford crude 3-iodo-5-*tert*-butylbenzyl bromide **17** (975 mg) as a colorless oil. The crude mixture also contains the starting material in approximately 40 mol% and corresponding dibromide in 10 mol% relative to the desired monobromide **17**. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.62 (s, 1H), 7.56 (s, 1H), 7.33 (s, 1H), 4.39 (s, 2H), 1.27–1.33 (m, 18H).

Step 2. To a solution of tetraisopropyl methylenediphosphonate (1.79 mL, 5.52 mmol) in dry THF (11 mL) was added *n*BuLi (1.55 M in hexane, 3.21 mL, 4.97 mmol) at -78 °C under argon atmosphere. After 10 min, a solution of **17** (975 mg) and TBAI (192 mg, 0.52 mmol) in dry THF (11 mL) was added dropwise. The reaction was stirred at room temperature for 24 h. Then water was added, and the solution was extracted with EtOAc and washed with brine, combined organic layers were dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (EtOAc/CH_2Cl_2 = 1:1 to EtOAc/CH_2Cl_2/MeOH = 10:10:1) to afford the title compound **18** (396 mg, 0.643 mmol, 13% over 2 steps) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.52 (t, *J* = 1.7, 1H), 7.43 (s, 1H),

7.21 (s, 1H), 4.78–4.69 (m, 4H), 3.12 (td, *J* = 16.1, 5.8 Hz, 2H), 2.45 (tt, *J* = 24.1, 6.3 Hz, 1H), 1.31–1.25 (m, 33H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 153.44, 142.10 (t, *J* = 7.2 Hz), 135.42, 132.63, 125.67, 94.29, 71.44 (t, *J* = 3.6 Hz, 2C), 71.18 (t, *J* = 3.6 Hz, 2C), 40.91 (t, *J* = 133.5 Hz), 34.78, 31.46 (t, *J* = 4.8 Hz), 31.30 (3C), 24.27 (4C), 23.99 (d, *J* = 2.4 Hz, 2C), 23.87 (d, *J* = 2.4 Hz, 2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 21.34 (s, 2P). HRMS–ESI (*m/z*): $[M+Na]^+$ calcd for $C_{24}H_{43}O_6P_2I$, 639,1427; found, 639.1445

Tetraisopropyl 2-(3-ethnyl-5-*tert*-butylphenyl)ethan-1,1-bisphosphonate (19). Step 1. To a solution of **19** (123.5 mg, 0.200 mmol) in THF (2.0 mL) was added TMS-acetylene (69.2 µL, 0.500 mmol), triethylamine (69.3 µL, 0.500 mmol), $PdCl_2(PPh_3)$ (11.2 mg, 0.016 mmol), and CuI (1.5 mg, 0.0080 mmol) at room temperature. After stirring for 24 h, water was added and the mixture was extracted with CH_2Cl_2. The organic layer was dried over Na_2SO_4, and concentrated under reduced pressure to give the crude TMS-protected intermediate as a brown oil (108.8 mg). ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.31 (s, 1H), 7.24 (s, 2H), 4.79–4.72 (m, 4H), 3.16 (td, *J* = 16.6, 6.3 Hz, 2H), 2.49 (tt, *J* = 24.1, 6.3 Hz, 1H), 1.31–1.24 (m, 33H), 0.23 (s, 9H).

Step 2. To a solution of the intermediate (108.8 mg) in MeOH (2.0 mL) was added K_2CO_3 (3.0 mg, 0.0217 mmol) at room temperature. After stirring for 24 h, water was added to the solution, and the mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (EtOAc/CH_2Cl_2 = 1:1 to EtOAc/CH_2Cl_2/MeOH = 10:10:1) to afford the title compound **19** (22.2 mg, 0.0431 mmol, 22% over 2 steps) as a pale pink oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.35 (s, 1H), 7.27 (s, 1H), 7.24 (s, 1H), 4.70–4.79 (m, 4H), 3.18 (td, *J* = 16.6, 6.3 Hz, 2H), 3.01 (s, 1H), 2.50 (tt, *J* = 24.1, 6.3 Hz, 1H), 1.37–1.27 (21H), 1.27-1.23 (m, 4H), 1.19 (s, 3H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 151.24, 140.02, 130.17, 127.29, 127.06, 121.41, 84.38, 76.23, 71.42 (d, *J* = 3.6 Hz), 71.10 (d, *J* = 7.2 Hz), 40.86 (t, *J* = 135.0 Hz), 34.70, 31.69 (t, *J* = 4.8 Hz), 31.31 (3C), 24.25 (4C), 23.99 (2C), 23.84 (2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 21.47 (s, 2P).

Tetraisopropyl 2-(3-azido-5-*tert*-butylphenyl)ethan-1,1-bisphosphonate (20)

To a solution of **18** (116.4 mg, 0.189 mmol) in EtOH (2.7 mL) and water (1.8 mL) was added NaN_3 (24.5 mg, 0.378 mmol), *trans*-N,N'-dimethylcyclohexane-1,2-diamine (0.045 mL, 0.028 mmol), CuI (3.6 mg, 0.019 mmol), and *L*-sodium ascorbate (1.9 mg, 0.0095 mmol), and then the solution was degassed by sonication under argon atmosphere. The solution was refluxed for 40 h, diluted with water, and extracted with $CHCl_3$ and EtOAc. The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (EtOAc/CH_2Cl_2/MeOH = 10:10:1) to afford the title compound **20** (75.3 mg, 0.142 mmol, 75%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.06 (s, 1H), 6.84 (s, 1H), 6.80 (s, 1H) 4.81–4.70 (m, 4H), 3.18 (td, *J* = 16.6, 6.3 Hz, 2H), 2.49 (tt, *J* = 36.7, 5.8 Hz, 1H), 1.31–1.25 (m, 33H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 153.19, 141.78, 139.41, 123.08, 116.93, 114.40, 71.22 (t, *J* = 3.6 Hz, 2C), 71.18 (t, *J* = 3.6 Hz, 2C), 40.86 (t, *J* = 133.5 Hz), 34.92, 31.46 (t, *J* = 4.8 Hz), 31.32 (3C), 24.27 (4C), 23.99 (d, *J* = 2.4 Hz, 2C), 23.87

(d, *J* = 2.4 Hz, 2C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 21.49 (s, 2P). ESI-MS (*m/z*): $[M + Na]^+$ 554.24.

3-Azido-5-ethynylbenzyl alcohol (22). A solution of methyl 3-azido-5-ethynylbenzoate **21**, which was prepared as previously described, [19] in dry THF (6 mL) was treated dropwise with diisobutylaluminium hydride (DIBAL) (1.0 M in hexane, 2.4 mL, 2.4 mmol) at 0 °C under argon atmosphere. After 1 h at the temperature, the reaction was quenched by sequentially adding MeOH, water, EtOAc, and 1M aqueous HCl. The organic layer was separated, washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography (hexane/EtOAc = 2:1) to afford the title compound **22** (17.3 mg, 0.100 mmol, 21%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.26 (s, 1H), 7.07 (s, 1H), 7.05 (s, 1H), 4.68 (s, 2H), 3.11 (s, 1H), 1.77 (br s, 1H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 143.07, 140.61, 126.74, 123.83, 121.50, 117.76, 82.42, 78.19, 64.22.

3-Azido-5-ethynylbenzyl bromide (23). A solution of **23** (17.0 mg, 0.0982 mmol) in $CHCl_3$ was treated dropwise with a solution of PBr_3 (0.1 mM in $CHCl_3$, 0.423 mL, 0.0423 mmol) at 0 °C under argon atmosphere, and the reaction was stirred at the temperature for 30 min. To the reaction was added an additional portion of PBr_3 (0.192 mmol), and the mixture was further stirred at room temperature for 30 min. The reaction was quenched with saturated aqueous $NaHCO_3$, and extracted with $CHCl_3$. The organic layer was washed with brine, dried over $MgSO_4$, and purified by flash column chromatography (hexane/EtOAc = 5:1) to afford the title compound **23** (20.6 mg, 0.0873 mmol, 89%) as a yellow oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.28 (t, *J* = 1.4 Hz, 1H), 7.08 (dd, *J* = 2.0, 1.4 Hz, 1H), 7.03 (t, *J* = 1.7 Hz, 1H), 4.40 (s, 2H), 3.13 (1H, s). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 140.85, 139.86, 129.06, 124.25, 122.28, 120.09, 81.95, 78.68, 31.57.

Tetraisopropyl 2-(3-azido-5-ethynylphenyl)ethan-1,1-bisphosphonate (24). To a solution of tetraisopropyl methylenediphosphonate (0.0890 mL, 0.274 mmol) in dry THF (2.0 mL) was added *n*BuLi (1.54 M in hexane, 0.16 mL, 0.25 mmol) at -78 °C under argon atmosphere. After 10 min, a solution of **23** (19.6 mg, 0.0830 mmol) and TBAI (6.5 mg, 0.018 mmol) in dry THF (2.0 mL) was added dropwise. The reaction was stirred at room temperature for 16 h. Then water was added, and the solution was extracted with EtOAc, and the organic layer was washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/CH_2Cl_2/MeOH = 100:100:0 to 100:100:5) to afford the title compound **24** (5.4 mg, 0.011 mmol, 13%) as a pale brown oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.19 (s, 1H), 6.98 (s, 1H), 6.93 (s, 1H), 4.65–4.74 (m, 4H), 4.09–4.13 (m, 1H), 3.15 (td, *J* = 16.3, 6.0 Hz, 2H), 3.06 (s, 1H), 2.43 (tt, *J* = 24.1, 6.0 Hz, 1H), 1.30-1.31 (m, 12H), 1.27 (d, *J* = 6.3 Hz, 6H), 1.25 (d, *J* = 6.3 Hz, 6H). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 21.04 (s, 2P).

Hydrogen triisopropyl 2-(2,6-di-*tert*-butylpyridine)ethan-1,1-bisphosphonate (25) To a solution of **SRP3042** (323.9mg, 0.591 mmol) in MeCN (1.0 mL) was added LiBr (51.4 mg, 0.591

mmol) at room temperature, and the solution was refluxed for 31 h. To the reaction was added 1 M aqueous HCl was added, and the mixture was extracted with CH_2Cl_2. The organic layer were washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel (EtOAc/CH_2Cl_2/MeOH = 5:5:1 to EtOAc/MeOH = 4:1) to afford the title compound **25** (120.4 mg, 0.238 mmol, 46%) as a white solid. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 6.93 (s, 2H), 4.91–4.87 (m, 1H), 4.55 (s, 1H), 4.41–4.35 (m, 1H), 3.34–3.23 (m, 1H), 2.95–2.86(m, 1H), 1.44–1.14 (m, 30H), 0.89 (d, *J* = 5.8 Hz, 3H), 0.84 (d, *J* = 5.7 Hz, 3H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 167.00 (2C), 149.59, 116.36 (2C), 71.97 (d, *J* = 6.0 Hz), 70.45 (d, *J* = 6.0 Hz), 68.17 (d, *J* = 4.8 Hz), 40.35 (t, *J* = 123.6 Hz), 37.48 (2C), 32.42, 30.25 (6C), 23.43–24.61 (m, 6C). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 27.63 (s, 1P), 12.71 (s, 1P).

Propargyl triisopropyl 2-(2,6-di-*tert*-butylpyridine)ethan-1,1-bisphosphonate (26). To a solution of compound **25** (64.1 mg, 0.127 mmol) in 1.0 mL CH_2Cl_2 was added oxalyl chloride (43.8 μL, 0.51 mmol) and DMF (4.0 μL, 0.051 mmol) at 0 °C, and the reaction was stirred for 30 min. Then the reaction was allowed to warm up to room temperature, and further stirred for 1 h. The solution was concentrated under reduced pressure to obtain a crude intermediate, which was used in the next reaction without further purification. To the solution of the intermediate in 1.5 mL CH_2Cl_2 was added 2-propyn-1-ol (15.0 μL, 0.254 mmol) and triethylamine (70.0 μL, 0.508 mmol) at 0 °C, and the reaction was stirred for 24 h at room temperature. To the reaction was added 1 M aqueous HCl, and the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (EtOAc/Hexane = 6:1) and preparative thin-layer chromatography (PTLC) (EtOAc/MeOH = 10:1, silica gel 60 PLC glass plate, 0.5 mm thickness from Merck Millipore) to afford the title compound (6.1 mg, 0.011 mmol, 8.8%) as a colorless oil. Note that NMR spectra indicated the presence of a pair of diastereomers with 1.3 : 1 ratio. The major isomer was marked as “a” and the minor one as “b” in the following spectra. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 6.98 (s, 2H, b), 6.97 (s, 2H, a), 4.54–4.85 (m, 5H+5H, a+b), 3.19–3.11 (m, 2H+2H, a+b), 2.72–2.58 (m, 1H+1H, a+b), 2.49 (t, *J* = 2.3 Hz, 1H, b), 2.47 (t, *J* = 2.3 Hz, 1H, a), 1.17–1.36 (m, 36H+36H, a+b). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 167.51 (s, 2C, b), 167.49 (s, 2C, a), 148.15 (d, *J* = 7.2 Hz, 1C, b), 148.05 (d, *J* = 7.2 Hz, 1C, a), 116.03 (s, 2C, b), 116.01 (s, 2C, a), 78.83 (d, *J* = 7.2 Hz, 1C, a), 78.56 (d, *J* = 7.2 Hz, 1C, b), 75.37 (s, 1C, b), 75.16 (s, 1C, a), 72.07 (d, *J* = 7.2 Hz, 1C, b), 71.90 (d, *J* = 7.2 Hz, 1C, a), 71.78 (d, *J* = 7.2 Hz, 1C, b), 71.57 (d, *J* = 7.2 Hz, 1C, a), 71.36 (d, *J* = 6.0 Hz, 1C, b), 71.27 (d, *J* = 7.2 Hz, 1C, a), 54.77 (d, *J* = 6.0 Hz, 1C, a), 54.08 (d, *J* = 6.0 Hz, 1C, b), 39.84 (t, *J* = 134.4 Hz, 1C, b), 39.74 (t, *J* = 134.4 Hz, 1C, a), 37.59 (s, 2C+2C, a+b), 31.54–31.43 (m, 1C+1C, a+b), 30.25 (s, 6C+6C, a+b), 23.77–24.35 (m, 6C+6C, a+b). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 24.00 (d, J = 6.2 Hz, 1P, a), 23.60 (s, 1P, b), 20.80 (d, *J* = 3.1 Hz, 1P, b), 20.65 (d, *J* = 4.7 Hz, 1P, a). HRMS–ESI (*m/z*): $[M + Na]^+$ calcd for $C_{27}H_{47}O_6NP_2$,566.2771; found,566.2770.

3-Butyn-1-yl triisopropyl 2-(2,6-di-*tert*-butylpyridine)ethan-1,1-bisphosphonate (27). To a solution of **25** (55.4 mg, 0.110 mmol) in 1.0 mL CH_2Cl_2 was added oxalyl chloride (37.7 μL, 0.44 mmol) and DMF (3.4 μL, 0.044 mmol) at 0 °C, and the reaction was stirred at the temperature for 30 min then at rt for 1 h. The solution was concentrated under reduced pressure, and the crude intermediat dissolved in 1.5 mL CH_2Cl_2 was treated with 3-butyn-1-ol (16.6 μL, 0.22 mmol) and triethylamine(61.0 μL, 0.44 mmol) at 0 °C. The reaction was stirred for 24 h at room temperature, and 1 M aqueous HCl was added to the solution. The mixture was extracted with EtOAc, and the organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by flash column chromatography (EtOAc/Hexane = 6:1) and PTLC (EtOAc/MeOH = 10:1) to afford the title compound **27** (5.7 mg, 0.010 mmol, 9.3%) as a colorless oil. Note that NMR spectra indicated the presence of a pair of diastereomers in 1:0.75 ratio. The major isomer was marked as “a” and the minor one as “b” in the following spectra. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 6.98 (s, 2H, b), 6.97 (s, 2H, a), 4.65–4.84 (m, 3H+3H, a+b), 4.07–4.23 (m, 2H+2H, a+b), 3.12–3.23 (m, 2H+2H, a+b), 2.69–2.56 (m, 2H+2H, a+b), 2.45–2.50 (m, 2H+2H), 1.96 (t, *J* = 2.6 Hz, 1H, b), 1.95 (t, *J* = 2.6 Hz, 1H, a), 1.20-1.35 (m, 36H+36H, a+b). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 167.51 (s, 2C+2C, a+b), 148.27 (t, *J* = 7.8 Hz, 1C+1C, a+b), 116.01 (s, 2C+2C, a+b), 80.10 (s, 1C, a), 79.98 (s, 1C, b), 71.27-71.80 (m, 3C+3C, a+b), 70.20 (s, 1C, b), 70.09 (s, 1C, a), 64.70 (d, *J* = 6.0 Hz, 1C, b), 64.09 (d, *J* = 7.2 Hz, 1C, a), 39.72 (d, *J* = 135.0 Hz, 1C, b), 39.58 (d, *J* = 135.0 Hz, 1C, a), 37.61 (s, 2C+2C, a+b), 31.65–31.60 (m, 1C+1C, a+b), 30.26 (s, 6C+6C, a+b), 23.77–24.30 (m, 6C+6C, a+b), 20.93 (d, *J* = 7.2 Hz, 2C+2C, a+b). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 22.90 (d, *J* = 6.0 Hz, 1P, a), 22.57 (d, *J* = 3.1 Hz, 1P, b), 21.05 (d, *J* = 3.1 Hz, 1P, b), 20.92 (d, *J* = 6.0 Hz, 1P, a). HRMS–ESI (*m/z*): $[M + Na]^+$ calcd for $C_{28}H_{49}O_6NP_2$,580.2927; found, 580.2917.

2-(3-But-3-ynyl-3H-diazirin-3-yl)-ethyl *p*-toluensulfonate (29). The title compound **29** was prepared as previously described. [42] 2-(3-But-3-ynyl-3H-diazirin-3-yl)ethanol **28** (22 μL, 0.172 mmol) was dissolved in 150 μL CH_2Cl_2 at 0 °C. After the addition of pyridine (41.6 μL, 0.516 mmol), Et_3N (71.5 μL, 0.516 mmol), and DMAP (1 mg) to the solution, a solution of *p*-toluenesulfonyl chloride (98.3 mg, 0.516 mmol) in 150 μL CH_2Cl_2 was added dropwise over 10 min, and the mixture was stirred at room temperature for 24 h. After addition of 1 M aqueous HCl, the mixture was extracted twice with diethyl ether. The organic layer was concentrated, and the residue was purified by flash column chromatography (hexane/EtOAc = 3:1) to afford the title compound (23.7 mg, 0.0811 mmol, 47%) as a yellow oil. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 7.81 (d, *J* = 8.3 Hz, 2H), 7.36 (d, *J* = 8.3 Hz, 2H), 3.90 (t, *J* = 6.3 Hz, 2H), 2.45 (s, 3H), 1.94–1.97 (m, 3H), 1.77 (t, *J* = 6.3 Hz, 2H), 1.60 (t, *J* = 7.2 Hz, 2H).

2-(3-But-3-ynyl-3H-diazirin-3-yl)ethyl triisopropyl 2-(2,6-di-*tert*-butylpyridine)ethan-1,1-bisphosphonate (srpDHY). Compound **25** (61.7 mg, 0.122 mmol) and tosylate **29** (23.7 mg, 0.081 mmol) were dissolved in a mixture of CH_2Cl_2 (0.60 mL) and DMF (0.60 mL), and Cs_2CO_3 (79.2 mg, 0.243 mmol) was added to the solution. The reaction mixture was stirred at 60 °C for 72 h and quenched with 1 M aqueous HCl. The mixture was extracted with EtOAc, and the

organic layer was washed with brine, dried over Na_2SO_4, and concentrated under reduced pressure. The crude product was purified by flash column chromatography (EtOAc/hexane = 6:1) to afford the title compound **srpDHY** (7.6 mg, 0.012 mmol, 15%) as a colorless oil. Note that NMR spectra indicated the presence of a pair of diastereomers in 1:0.75 ratio. Although the separation of the major/minor isomers was unclear and their assignment can be ambiguous, the major isomer was tentatively marked as "a" and the minor one as "b" in the following spectral data, where possible. ^{1}H-NMR ($CDCl_3$, 500 MHz, δ): 6.99 (s, 2H, b), 6.98 (s, 2H, a), 4.67–4.81 (m, 3H+3H, a+b), 3.92–4.02 (m, 2H+2H, a+b), 3.11–3.20 (m, 2H+2H, a+b), 2.57–2.67 (m, 1H+1H, a+b), 2.00 (td, *J* = 7.6, 2.7 Hz, 2H, b), 1.99 (td, *J* = 7.6, 2.7 Hz, 2H, a), 1.95 (t, *J* = 2.7 Hz, 1H, a), 1.94 (t, *J* = 2.7 Hz, 1H, b), 1.64-1.77 (m, 4H+4H, a+b), 1.20–1.34 (m, 36H+36H, a+b). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ): 167.52 (s, 2C+2C, a+b), 148.18–148.36 (m, 1C+1C, a+b), 116.00 (s, 2C+2C, a+b), 82.63 (s, 1C+1C, a+b), 71.24–71.92 (m, 3C+3C, a+b), 69.37 (s, 1C, a), 69.32 (s, 1C, b), 61.70 (d, *J* = 6.0 Hz, 1C, b), 61.13 (d, *J* = 6.0 Hz, 1C, a), 39.70 (t, *J* = 134.4 Hz, 1C, a), 39.54 (t, *J* = 134.4 Hz, 1C, b), 37.61 (s, 2C+2C, a+b), 34.20 (s, 1C, a), 34.14 (s, 1C, b), 32.24 (s, 1C, a), 32.21 (s, 1C, b), 31.58-31.67 (m, 1C+1C, a+b), 30.27 (s, 6C+6C, a+b), 26.24 (s, 1C, a), 26.22 (s, 1C, b), 23.72–24.28 (m, 6C+6C, a+b), 13.33 (s, 1C+1C, a+b). ^{31}P-NMR ($CDCl_3$, 202 MHz, δ): 23.03 (d, *J* = 4.7 Hz, 1P, a), 22.75 (d, *J* = 3.5 Hz, 1P, b), 20.97 (d, *J* = 3.5 Hz, 1P, b), 20.87 (d, *J* = 4.7 Hz, 1P, a). HRMS–ESI (*m/z*): $[M + Na]^+$ calcd for $C_{31}H_{53}O_6N_3P_2$, 648.3302; found, 648.3295. Copies of the NMR spectra can be found in the Supplementary Materials.

2.4 Luciferase-based HMGCR degradation assay

HMGCR degradation activity of SR12813 derivatives was tested using the HEK293 cell line stably expressing HMGCR-dCat-ELuc, as previously described. [34] Briefly, the HMGCR-dCat-ELuc expressing cells cultured on a white 96-well clear-bottom plate (Greiner, product number 655098) were treated with test compounds for 4 h, and the remaining amount of HMGCR-dCat-ELuc proteins were quantified using luciferase assay. For each experiment, luminescence data was normalized with vehicle (0.1% DMSO)-treated samples and expressed as % of control. To confirm that the compound treatment does not compromise cell viability, CytoRed viability assay, which detects cellular esterase activity, was performed from the same samples within the wells just before luciferase activity measurement. [43] The compounds at the concentrations tested did not show cytotoxicity unless otherwise noted.

2.5 Photoaffinity labeling of HMGCR-dCat-ELuc and Insig

The protocol for the photoaffinity labeling experiments were adopted from our previous report and modified slightly. [19,44] HEK293 cells stably expressing HMGCR-dCat-ELuc were cultured to about 70% confluency, and transiently transfected with pCMV-Insig1-myc-DDK using Lipofectamine 3000 following the manufacturer's instructions. After 6 h, the culture medium was replaced with fresh medium with additional 6 μM compactin, which was added to increase the

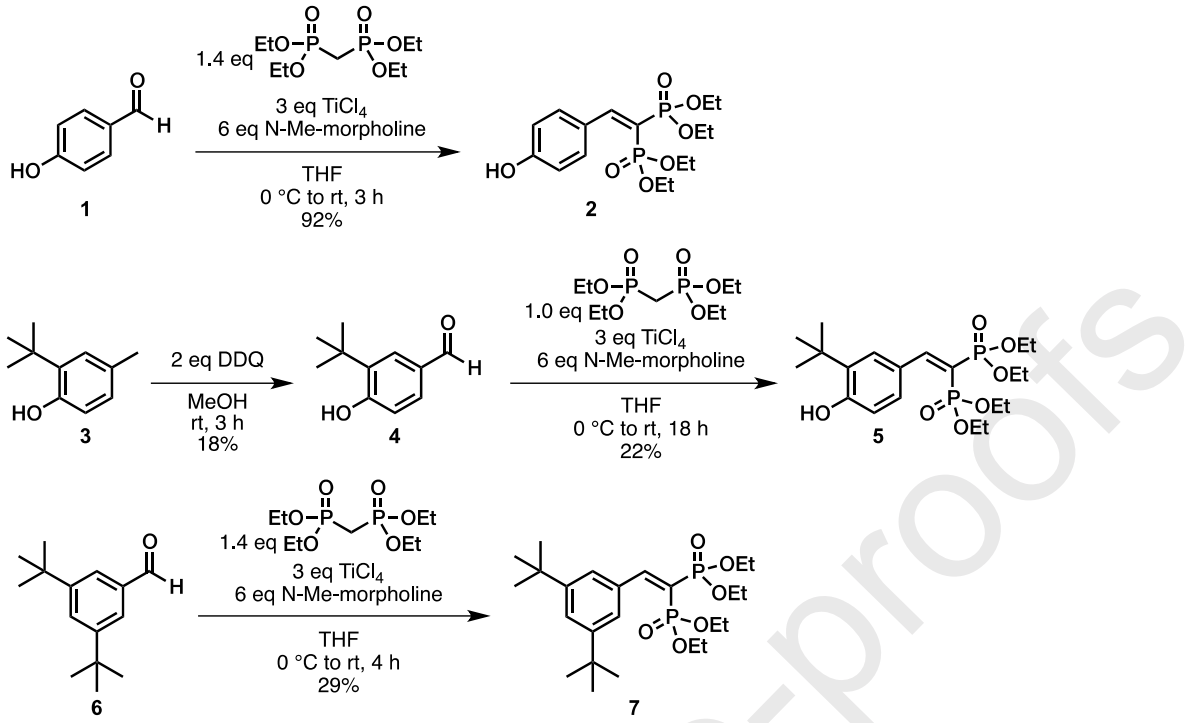
expression level of HMGCR-dCat-ELuc. After 16 h of compactin treatment, cells were scraped into ice-cold PBS and membrane preparation was performed as described previously. [19,44] The membrane fraction was treated with 20 μM **srpDHY** for 30 min on ice in the absence or presence of a competitor **SRP3042**, and irradiated with UV light (approximately 1 cm from the sample, 365 nm, LED365-SPT/L from OptoCode, Tokyo, Japan) for 3 min on ice. The membrane fractions were then solubilized and denatured with 1% SDS and 1% Triton X-100, and biotin was conjugated onto the alkyne on the **srpDHY** via copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. [45] The reaction mixtures were diluted 10-fold and subjected to anti-FLAG immunoprecipitation with anti-FLAG M2 magnetic beads (4 °C, overnight). The beads were washed, and eluted with a mixture of TNET, membrane protein solubilization buffer, (62.5 mM Tris pH 6.8, 8 M urea, 100 mM DTT, 15% SDS, 10% glycerol), and Laemmli sample buffer (2:2:1) at 37 °C for 30 min. [33,34,46,47] The eluted samples were then resolved on SDS-PAGE (SuperSep Ace 10-20% gel, FUJIFILM-Wako Pure Chemicals), and proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore). The membrane was probed with ImmunoPure streptavidin-HRP (Pierce, 1/12000 dilution in TBST) or anti-FLAG M2 antibody (Sigma-Aldrich, 1/2000 dilution in CanGetSignal signal enhancer solution) as previously described. [44]

3. Results

3.1 Pharmacophore analysis of SR12813 derivatives as HMGCR degraders

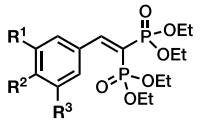
Although the rational design of photoaffinity probes from **SR12813** requires detailed SAR data regarding HMGCR degradation activity, no direct comparison of **SR12813** derivatives on HMGCR degradation activity has been performed until now, and only limited information on cholesterol synthesis inhibitory activity is available. [29] Therefore, we started to evaluate SAR of **SR12813** derivatives on HMGCR degradation activity, aiming at rationally designing photoaffinity probes.

First, to gain insight into where we could modify and append functional groups required for photoaffinity labeling probes, we examined the requirement of the bulky *tert*-butyl groups and the phenolic hydroxyl group on **SR12813** (Scheme 1 and Table 1). Removal of either one or both of the *tert*-butyl groups resulted in the complete loss of HMGCR degradation activity (compare **SR12813**, **2**, and **5**), clearly indicating the importance of these *tert*-butyl groups. In contrast, the phenolic hydroxyl group was dispensable, as exemplified by the comparable activity of the compound **7** and **SR12813**. Although the dispensability of the hydroxyl group suggested a possibility that we can introduce some functional groups on it, our attempts to alkylate or acylate the hydroxyl group failed to give sufficient conversion of **SR12813**.



Scheme 1. Synthesis of SR12813 derivatives listed in Table 1

Table 1. Requirement of the *tert*-butyl groups and dispensability of the phenolic hydroxyl group for HMG-CoA reductase (HMGCR) degradation activity

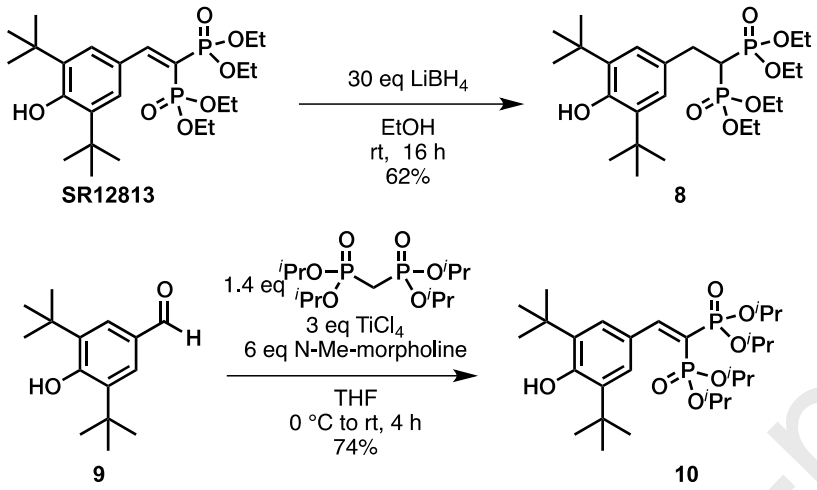


Compounds	R^1	R^2	R^3	EC_{50} (μM) (mean ± SD)[a]
SR12813				1.10 ± 0.35
2				NA[b]
5				NA[b]
7				1.16 ± 0.15

[a]The EC_{50} values represent mean ± SD from more than three independent dose-response experiments.
[b]NA, no activity at 10 μM.

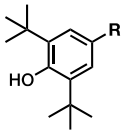
Next, we examined the importance of the double bond in **SR12813** and alkyl groups (Et or iPr) on the bisphosphonate esters. Of the four combinations tested (Scheme 2 and Table 2), **SR45023A**, which lacks the double bond and has the iPr group on the esters, was found to

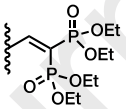
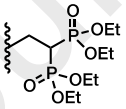
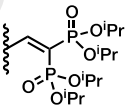
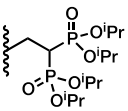
possess slightly more potent HMGCR degradation activity than **SR12813**. At higher concentrations, the bisphosphonates with the double bond tended to be slightly more toxic than their counterparts with the single bond. Therefore, we selected **SR45023A** (single bond and iPr esters) as a new starting point for the next round of SAR examination.



Scheme 2. Synthesis of **SR12813** derivatives listed in Table 2

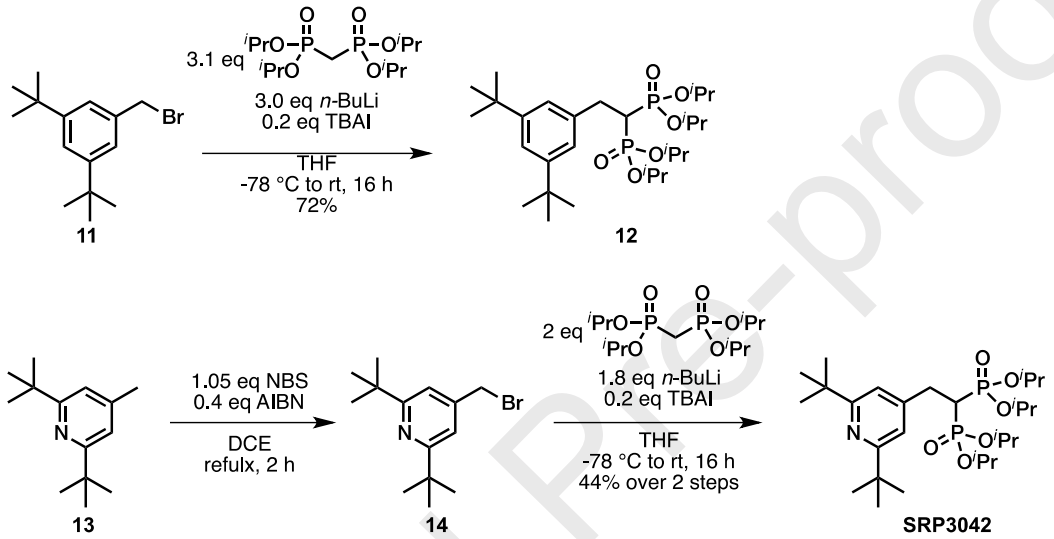
Table 2. HMG-CoA reductase (HMGCR) degradation activity of bisphosphonate esters with double or single bond and with Et or iPr esters



Compounds	R	EC_{50} (μM) (mean ± SD)[a]
SR12813		1.10 ± 0.35
8		2.77 ± 0.25
10		1.34 ± 0.14
SR45023A		0.876 ± 0.086

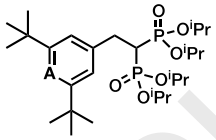
[a]The EC_{50} values represent mean ± SD from more than three independent dose-response experiments.

Considering the dispensability of the phenolic hydroxyl group on **SR12813**, we removed the hydroxyl group from **SR45023A** to find 3-fold improvement in activity (compound **12** in Scheme 3 and Table 3). Furthermore, when the aromatic C-H was replaced with pyridine nitrogen (Table 3, **SRP3042**), an additional 2-fold improvement in HMGCR degradation activity was observed. Although the reason for the improvement is not clear at this point, differences in the hydrogen bond donor/acceptor property, steric effect, or electronic effect on the aromatic ring might contribute to this improvement. As far as the authors are aware, this nitrogen-containing bisphosphonate ester **SRP3042** is the most potent HMGCR degrader reported to date.



Scheme 3. Synthesis of **SRP3042**, an **SR45023A** derivative with a pyridine nitrogen

Table 3. Removal of the phenolic hydroxyl group and introduction of nitrogen into **SR45023A** improves HMG-CoA reductase (HMGCR) degradation activity

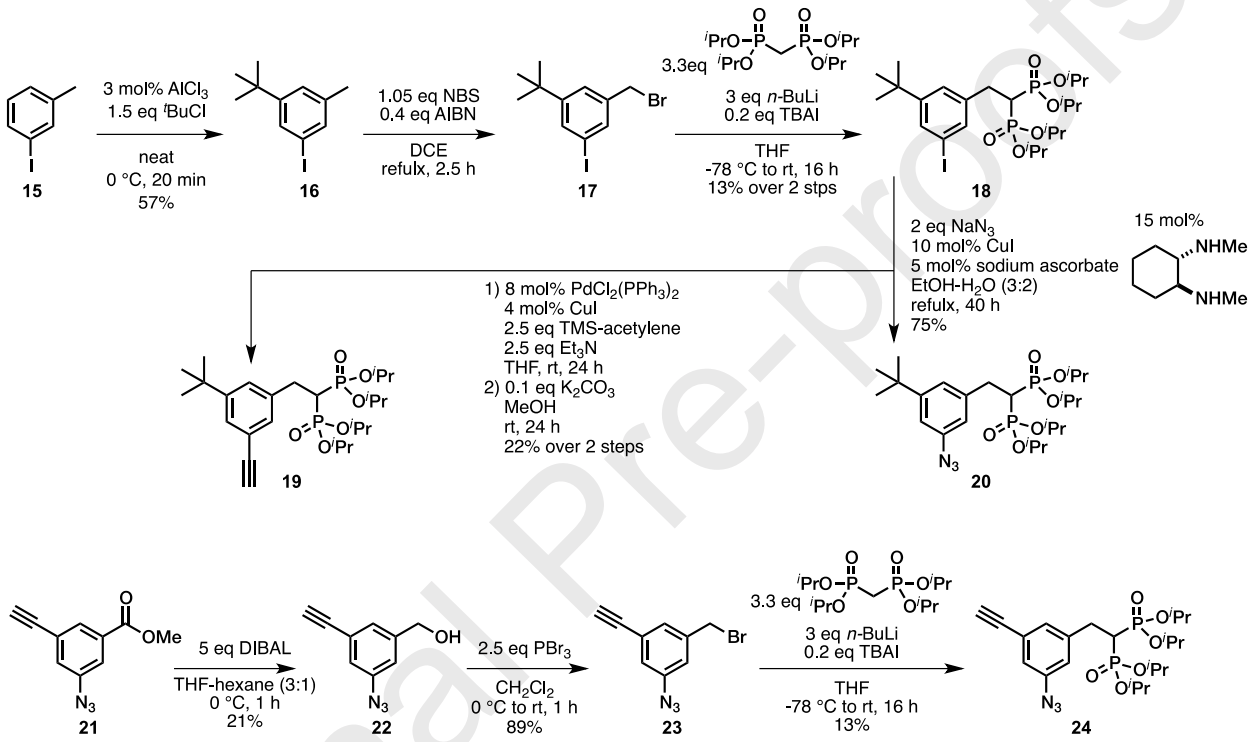


Compounds	A	EC_{50} (μM) (mean ± SD)[a]
SR45023A		0.876 ± 0.086
12		0.317 ± 0.106
SRP3042		0.153 ± 0.032

[a]The EC_{50} values represent mean ± SD from more than three independent dose-response experiments.

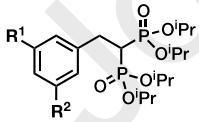
3.2 Photoaffinity bisphosphonate esters with HMGCR degradation activity

As is clear from the initial analysis shown in Table 1, the presence of the *tert*-butyl group was essential for the HMGCR degradation activity. Here, we examined if replacement of one or both of the *tert*-butyl groups with an ethynyl group or an azide, which can serve as a clickable handle or photo-reactive group, can be tolerated. As shown in Scheme 4 and Table 4, replacement of one *tert*-butyl group on compound **12** resulted in 4- to 11-fold lower activity (**12**, **18**-**20**), and replacement of both groups gave an inactive compound **24**.



Scheme 4. Synthesis of the bisphosphonate ester 6 derivatives with *tert*-butyl group(s) replaced with probe units, an alkyne and/or an azide

Table 4. Attempts to introduce functional groups required for photoaffinity probes at the *tert*-butyl groups

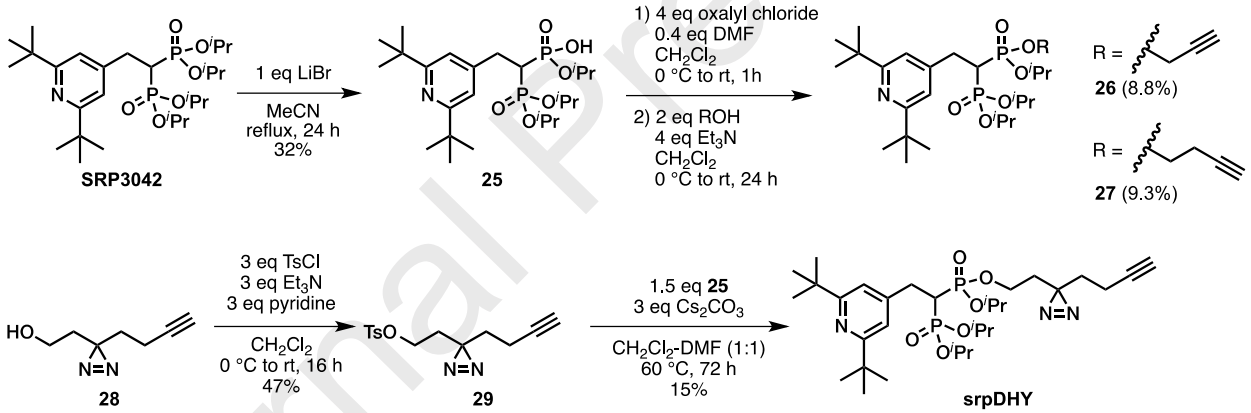


Compounds	R^1	R^2	EC_{50} (μM) (mean ± SD)[a]
12			0.317 ± 0.106
18			1.46 ± 0.30

19			3.54 ± 1.45
20			1.66 ± 0.38
24			NA[b]

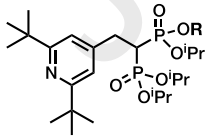
[a]The EC_{50} values represent mean ± SD from more than three independent dose-response experiments.
[b]NA, no activity at 10 μM.

We next focused our attention on an alkyl group on the bisphosphonate esters, and introduced alkyne-containing alkyl groups or a minimalist photo-crosslinker that contains both diazirine, a photo-reactive group, and a terminal alkyne (Scheme 5 and Table 5). [48] Modifications at one of the phosphonate ester were relatively well tolerated (4- to 6-fold loss of activity for compounds **25**, **26**, and **27**), and we obtained a photoaffinity probe **srpDHY** with minimal loss of HMGCR degradation activity (3-fold loss). The loss owing to modification of the alkyl group was compensated by the improved activity of **SRP3042**, overall giving a more potent bisphosphonate ester **srpDHY** than the original **SR12813** or **SR45023A** (Fig. 2A).

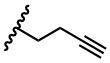
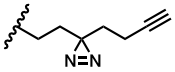


Scheme 5. Synthesis of **SRP3042** derivatives with probe units introduced on one of the phosphonate esters

Table 5. Effect of modifications on the phosphonate ester moiety and identification of a photoaffinity probe **srpDHY**



Compounds	R	EC_{50} (μM) (mean ± SD)[a]
25		NA[b]

SRP3042		0.153 ± 0.032
26		0.633 ± 0.150
27		0.795 ± 0.257
srpDHY		0.417 ± 0.052

[a]The EC_{50} values represent mean ± SD from more than three independent dose-response experiments.
[b]NA, no activity at 10 μM.

3.3 Direct interaction of the bisphosphonate esters with the membrane domain of HMGCR

With the potent photoaffinity probe **srpDHY** in hand (Fig. 2A), we set out to test the interaction between the bisphosphonate and HMGCR or Insig. The membrane fraction was isolated from the cells expressing both HMGCR-dCat-ELuc tagged with the FLAG tag and Insig1 tagged with myc and FLAG tags, and the membrane fraction was labeled with the **srpDHY** probe in the absence or presence of a competitor, **SRP3042**. After immunoprecipitation of both the FLAG-tagged HMGCR-dCat-ELuc and Insig1, the extent of **srpDHY**-labeling was examined via biotin introduced on the alkyne through CuAAC reaction (Fig. 2B). UV irradiation resulted in selective labeling of the HMGCR membrane domain over Insig1, and the extent of the labeling was reduced by competition with **SRP3042**, supporting specific interaction between the bisphosphonate ester and the HMGCR membrane domain.

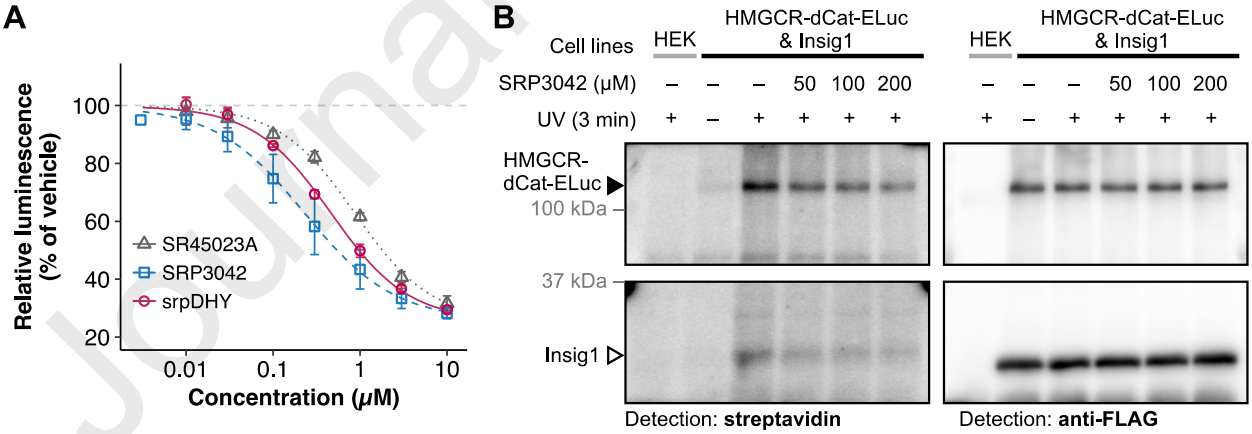


Figure 2. Photoaffinity labeling of HMGCR and Insig with the **srpDHY** probe. (A) Dose-dependent degradation of HMGCR by **SRP3042** and **srpDHY** probes, in comparison with that by **SR45023A**. Data represent mean ± SD of more than three independent dose-response experiments. (B) Photoaffinity labeling of HMGCR-dCat-ELuc and Insig1 by srpDHY probe and its competition by **SRP3042**. Cells stably expressing HMGCR-dCat-FLAG-ELuc were transiently transfected with pCMV-Insig1-myc-FLAG, and the membrane fraction was isolated after pre-

treatment with 6 μM compactin for 16 h to facilitate detection of HMGCR. The membrane fraction was labeled with **srpDHY** with or without competition from **SRP3042**, and biotin was introduced onto the alkyne via click chemistry. After immunoprecipitation of both HMGCR-dCat-FLAG-ELuc and Insig1-myc-FLAG, the presence of biotin and FLAG tag was probed.

4. Discussion

In this study, we identified a potent photoaffinity probe **srpDHY** based on a more potent **SR12813**/**SR45023A** derivative **SRP3042** and evaluated its binding to the HMGCR membrane domain. Our data showed that the bisphosphonate esters preferentially interacted with the HMGCR membrane domain over co-expressed Insig1. Although Insig1 was also slightly labeled with **srpDHY** in a UV- and competition-dependent manner, a most likely interpretation would be that complex formation of HMGCR and Insig1 brought the proteins in close proximity, within which the photo-activated probe labeled the other protein. Alternatively, we cannot exclude a possibility that the bisphosphonate esters bind to the interface of the HMGCR and Insig1, resulting in labeling of both proteins, with the limited spatial resolution of the photoaffinity labeling technique. Further studies would be needed to clarify the remaining questions: where the bisphosphonate esters binds to within the membrane domain of HMGCR, how the compound is recognized, and how the binding of the bisphosphonate esters triggers the sequence of events that leads to degradation of HMGCR.

Statins have remained to be effective drugs in reducing serum cholesterol level since their discovery in the early 1970s. [1,49] However, prolonged use of statins sometimes results in massive increase in HMGCR protein level through SREBP pathway activation and cancellation of sterol-induced degradation. [15,25] Such compensatory increase gives rise to statin resistance, necessitates increased dose of statins, and concomitantly leads to increased risk of their side effects. [47] The increased level of HMGCR also worsens the clinical outcome if statin treatment is interrupted or discontinued, a phenomenon called rebound or withdrawal syndrome. [50] Therefore, the combined use of HMGCR degraders along with statins is expected to be synergistically effective in reducing serum cholesterol, decreasing atherosclerosis, and reducing the risk associated with the statin-induced upregulation of HMGCR proteins. [47]

Additionally, a form of immune-mediated necrotizing myopathy, which has been linked to statin use, is characterized by the presence of autoantibodies against HMGCR and is reported to be progressive even after discontinuation of statin treatment. [51] HMGCR degraders might be useful in reducing HMGCR proteins and thereby suppressing immune reactions against HMGCR, although this possibility needs to be experimentally validated.

Overall, through preliminary SAR study starting from **SR12813**, we identified a more potent HMGCR degrader **SRP3042** and developed a potent photoaffinity probe **srpDHY**. Labeling experiments with the probe indicated direct binding of the bisphosphonate esters to the membrane domain of HMGCR, augmenting our mechanistic understanding of the bisphosphonate ester-mediated degradation of HMGCR. Additionally, our finding of **SRP3042**, the most potent HMGCR degrader reported to date, offers a promising avenue to find better

small molecules that may lead to improved treatment of hypercholesterolemia, atherosclerosis, and immune-mediated necrotizing myopathy.

Acknowledgements

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: